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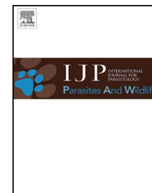
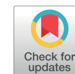


Comparison of the modified agglutination test and real-time PCR for detection of *Toxoplasma gondii* exposure in feral cats from Phillip Island, Australia, and risk factors associated with infection



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ABSTRACT

Toxoplasma gondii is considered a disease risk for many native Australian species. Feral cats are the key definitive host of *T. gondii* in Australia and therefore, investigating the epidemiology of *T. gondii* in cat populations is essential to understanding the risk posed to wildlife. Test sensitivity and specificity are poorly defined for diagnostic tests targeting *T. gondii* in cats and there is a need for validated techniques. This study focused on the feral cat population on Phillip Island, Victoria, Australia. We compared a novel real-time PCR (qPCR) protocol to the modified agglutination test (MAT) and used a Bayesian latent class modelling approach to assess the diagnostic parameters of each assay and estimate the true prevalence of *T. gondii* in feral cats. In addition, we performed multivariable logistic regression to determine risk factors associated with *T. gondii* infection in cats. Overall *T. gondii* prevalence by qPCR and MAT was 79.5% (95% confidence interval 72.6–85.0) and 91.8% (84.6–95.8), respectively. Bayesian modelling estimated the sensitivity and specificity of the MAT as 96.2% (95% credible interval 91.8–98.8) and 82.1% (64.9–93.6), and qPCR as 90.1% (83.6–95.5) and 96.0% (82.1–99.8), respectively. True prevalence of *T. gondii* infection in feral cats on Phillip Island was estimated as 90.3% (83.2–95.1). Multivariable logistic regression analysis indicated that *T. gondii* infection was positively associated with weight and this effect was modified by season. Cats trapped in winter had a high probability of infection, regardless of weight. The present study suggests qPCR applied to tissue is a highly sensitive, specific and logistically feasible tool for *T. gondii* testing in feral cat populations. Additionally, *T. gondii* infection is highly prevalent in feral cats on Phillip Island, which may have significant impacts on endemic and introduced marsupial populations.

1. Introduction

Toxoplasma gondii is a globally distributed protozoan parasite. Cats, and other members of the family Felidae, are the only known definitive hosts (Dubey, 2010). Any warm-blooded animal may become infected with *T. gondii* via one of three pathways: environmental exposure (ingestion of oocysts shed into the environment by cats), carnivory (ingestion of *T. gondii* tissue cysts within an intermediate host), and vertical transmission (Dubey, 2010; Parameswaran et al., 2009; Prestrud et al., 2007). Toxoplasmosis, clinical disease due to *T. gondii*, is considered to be a significant cause of morbidity and mortality in Australian native wildlife (Bettiol et al., 2000; Obendorf and Munday, 1990; Portas, 2010). In Australia, the only felid species is the introduced cat (*Felis catus*) and feral cats occur across 99.8% of Australia's land area (Legge et al., 2017). Therefore, feral cats play a critical role in the epidemiology of toxoplasmosis in the Australian landscape.

The present study was initiated following a comprehensive disease risk analysis process assessing the suitability of Phillip Island, Victoria, Australia, as a release site for the critically endangered eastern barred bandicoot (*Perameles gunnii*) (Jakob-Hoff et al., 2016). Toxoplasmosis

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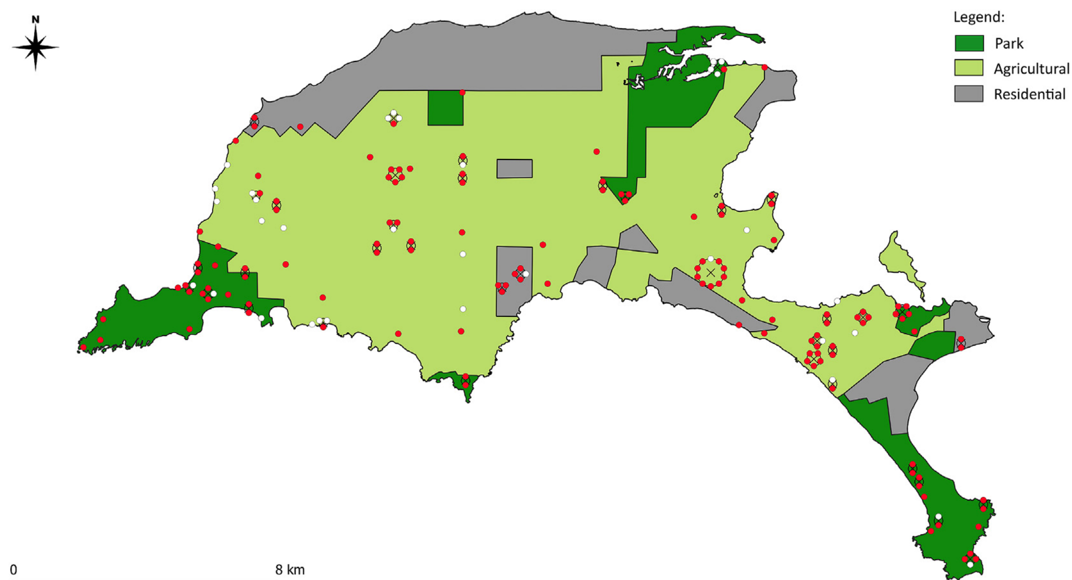

Fig. 1. Location and *Toxoplasma gondii* infection status, as detected by real-time PCR (qPCR), of feral cats trapped on Phillip Island (Victoria) from July 2016 to December 2017. Map shows the distribution of different location types (Park, Agricultural, Residential) used in multivariable regression analysis. A circular spread of points around a location marked with 'x' indicates multiple animals were sampled at the same site (i.e. same GPS coordinates). Red = *T. gondii* qPCR positive, white = *T. gondii* qPCR negative. Map created using Quantum GIS, version 3.8. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

was identified as a key threat to the successful establishment of this species, which is known to be fatally susceptible (Bettiol et al., 2000; Groenewegen et al., 2017). Furthermore, Phillip Island is a significant ecotourism destination and provides habitat for several iconic native species, such as the Australian fur seal (*Arctocephalus pusillus*) and the little penguin (*Eudyptula minor*). These species, and others inhabiting the island, may also be susceptible to toxoplasmosis (Donahoe et al., 2014; Mason et al., 1991). Understanding the epidemiology of *T. gondii* in feral cats on Phillip Island is important for determining if mitigation measures are required to ensure the success and sustainability of present and future translocations of endangered native fauna.

Cats maintain life-long antibodies to *T. gondii* after seroconversion (Dubey et al., 1995), and a variety of different serological tests have been developed to detect exposure. The modified agglutination test (MAT) is considered a highly sensitive and specific test for the detection of anti-*T. gondii* IgG (Desmonts and Remington, 1980). Macri et al. (2009) estimated the diagnostic sensitivity and specificity of the MAT in cats as 97.8% and 100%, respectively. However, these parameters were calculated using the indirect fluorescent antibody test (IFAT) as the reference, which has an estimated sensitivity and specificity of 81.0% and 93.8% (Dabritz et al., 2007). Comparison to an imperfect test leads to inaccurate estimation of test parameters, and these values should therefore be interpreted with caution.

PCR can be used to detect *T. gondii* DNA in animal tissues for the diagnosis of infection and is considered to be both highly specific and highly sensitive (Belaz et al., 2015). Given the variation in tissue predilection of *T. gondii* between hosts, and the heterogeneous distribution of bradyzoite cysts within tissues, selection of tissue(s), quantity of tissue and sampling technique all influence test sensitivity (Belaz et al., 2015; Hill et al., 2006). PCR can be used in situations when sera are not available, or antibody preservation is compromised, for example in wildlife studies when scavenged carcases are the most readily available sample set. Furthermore, PCR-based techniques may be expanded to allow sequencing and strain typing, which may be important in detailed epidemiological studies.

There is no perfect reference test for the detection of *T. gondii* (i.e. 100% specificity and 100% sensitivity). Therefore, despite the abundance of diagnostic tests available, the sensitivities and specificities of these tests remain poorly defined in cats. Accurate estimation of these test parameters is essential to inform high-quality epidemiological studies. Bayesian latent class (BLC) analysis can be used to determine test sensitivity and specificity in the absence of sufficient reference samples (i.e. when only imperfect tests are available), and this approach is the current World Organisation for Animal Health (OIE) recommendation for validation of these parameters (Branscum et al., 2005; OIE, 2014). Key advantages of BLC analyses are that no assumption is made about the true disease state of an animal, and prior knowledge may be incorporated into the model to better inform the outputs (Kostoulas et al., 2017). In the present study, we used BLC analysis to compare two different testing methods for detection of *T. gondii* in the cat: a TaqMan based real-time PCR (qPCR) protocol using DNA extracted from proteinase K digested tissues (direct detection), and the MAT (indirect detection). Furthermore, we aimed to determine the risk factors associated with *T. gondii* infection in feral cats on Phillip Island, to further elucidate the mechanisms of parasite transmission in this ecosystem, and to attempt to gain greater insight into the risks *T. gondii* poses to native Australian species at this site.

2. Materials and methods

2.1. Study site and sampling strategy

Phillip Island is a 10,000-ha island located in Westernport Bay, Victoria, Australia, and has been estimated to contain > 8,000 ha of suitable habitat for *P. gunnii* (Coetsee, 2016; Parrott et al., 2017). The island has a significant feral cat population, which was estimated at 3.4 cats/km^2 at the time of the present study (Legge et al., 2017). *Toxoplasma gondii* infection was presumed to be present in feral cats on Phillip Island, but had not been previously reported (D Sutherland, personal communication). A minimum sample size of 196 was calculated to be required to estimate the diagnostic sensitivity and specificity of the MAT and qPCR (based on an estimated diagnostic test specificity of 0.85), and the true prevalence of *T. gondii* infection in cats (based on an expected prevalence of 0.90), with ± 5% absolute precision and 95% confidence (Stevenson et al., 2018). Samples were collected opportunistically from feral cats euthanased under animal control

programs carried out by authorised officers (authorised under: *Catchment and Land Protection Act 1994* (Vic)) on Phillip Island between July 2016 and December 2017. Cats were collected by various means, including live trapping followed by euthanasia, shooting and collection of roadkill carcases. Trapping effort was constant across the year, and traps were well distributed across the island with a higher density of traps deployed within conservation reserves (Fig. 1).

2.2. Tissue and blood collection

Blood was collected by pest control rangers via cardiac puncture post-mortem, placed into serum gel separator tubes, centrifuged for 10 min and serum collected. Serum and carcases were stored at −20 °C prior to processing. Demographic data, including location, weight, and sex were recorded for each animal. The ages of cats could not be estimated using an objective measure and were therefore not used in the analysis.

2.3. Modified agglutination test (MAT)

The MAT was performed using a commercially available kit (*Toxo-Screen* DA, bioMerieux, Mercy l'Etoile, France), as per Obendorf et al. (1996) with minor modifications. Modified agglutination tests were titrated out to a maximum dilution of 1/256 for each sample i.e. final end-point titres were not determined. Briefly, 25 μl of sample serum was diluted in 175 μl of phosphate-buffered saline (PBS), and vortexed well to mix, before performing two 1/4 serial dilutions in PBS. Positive and negative control sera were supplied with the kit and used at a dilution of 1/8. 25 μl of each sample dilution, and controls, was transferred to a round-bottom 96-well microtitre plate, and 25 μl of 2-mercaptoethanol was added to each well, resulting in final test serum dilutions of 1/16, 1/64, and 1/256, and 1/16 for the positive and negative control. 25 μl of kit-supplied antigen (formalin treated T. gondii tachyzoites) at 1/4 dilution was added to each well, and the plate shaken gently to mix. The plate was covered with adhesive-backed plastic (kit supplied), or placed within a zip-lock bag, to prevent evaporation and incubated at room temperature for 6 h. The reaction was considered positive when a layer of agglutinated tachyzoites covering more than half of the base of the well was observed. The reaction was considered negative when sedimentation of the tachyzoites formed a small button in the base of the well. Individuals with a titre of ≥1/64 were considered T. gondii seropositive. This cut-off value is consistent with other studies on feral cats in Australia and therefore allowed direct comparison between these studies (Adams, 2003; Fancourt and Jackson, 2014). The same observer performed all tests. Results were converted to a binary variable (positive or negative) for analysis.

2.4. Tissue digestion and DNA extraction

Each carcase was defrosted at room temperature overnight and tissues collected the following day. Tissues were either processed immediately or stored at −20 °C. A total of 5 g of tissue, consisting of 1 g each of brain, skeletal muscle (*biceps femoris*) and heart, and 2 g of tongue, was collected from each carcase. For 31 cats, liver and lung (1 g each) was also collected, and a total of 7 g of tissue was processed. Artificial tissue digestion and DNA extraction was carried out as per Cuttell et al. (2012) with minor adaptations as follows: Tris-EDTA lysis buffer (40 mM Tris, 10 mM EDTA; Sigma-Aldrich, St. Louis, Missouri, USA) was added to pooled tissue to make up to a total volume of 10 ml and incubated in a water bath at 90 °C for 10 min. Samples were cooled to room temperature before the addition of 4 mg of Proteinase K (Bioline, London, UK). The samples were incubated overnight at 55 °C and thoroughly homogenised using a tissue homogeniser (T10 Basic Tissue Homogeniser, IKA-Werke, Staufen im Breisgau, Germany). DNA was extracted from the homogenised tissue lysate using either the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), or ISOLATE II Genomic DNA extraction kit (Bioline). After homogenisation, 300 μl of crude lysate was transferred to a 1.5 ml microcentrifuge tube and 300 μl of tissue lysis buffer (Qiagen Buffer AL, or Bioline Buffer G3) was added before incubation at 70 °C for 10 min. The sample was centrifuged at 14,000 rpm to pellet cellular debris, and 400 μl of supernatant was removed to a clean 1.5 ml microcentrifuge tube containing 210 μl of ethanol. At this point, all supernatant was added to the extraction column, and the protocol was followed as per kit instructions. All DNA extractions were assessed for total nucleic acid concentration and purity using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA extraction of the crude tissue lysate was repeated for any samples with significant contaminants likely to affect downstream applications.

2.5. Real-time polymerase chain reaction

Genomic DNA extracts were subjected to real-time PCR (qPCR) as described by Lelu et al. (2011) with minor modifications. Reactions were carried out on a MIC Personal qPCR Cycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia). The 529 bp highly repetitive element (GenBank AF 487550) was targeted by the TaqMan probe 5′-6FAM-ACGCTTTCCTCGTGGTGATGGCG-IBFQ-3′ and the DNA oligonucleotide primers 5′-AGAGACACCGGAATGCGATCT-3′ and 5′-CCCTCTTCTCCACTCTTCAATTCT-3'. As an internal control for PCR inhibition, Equine Herpes Virus (EHV) DNA was added to the reaction mix and targeted by the TaqMan probe 5′-HEX-ACGCTTTCCTCGTGG TGATGGCG-IBFQ-3′ and the DNA oligonucleotide primers 5′-GAT GACACTAGCGACTTCGA-3′ and 5′-CAGGGCAGAAACCATAGACA-3'. DNA oligonucleotide primers and probes were synthesized by Integrated Gene Technologies, Singapore. The amplification mixture consisted of 10 μl of GoTaq qPCR Master Mix (Promega, Madison, Wisconsin, United States), 150 nM of each *T. gondii* primer, 150 nM of the *T. gondii* TaqMan probe, 40 nM of each EHV primer, 100 nM of the EHV TaqMan probe, 1 μl of EHV template DNA, and 2 μl of sample genomic DNA, in a final reaction volume of 20 μl. The reaction mixture was subjected to an initial incubation of 95 °C for 2 min, and then 45 cycles of: denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 60 s. Negative and positive controls were included in each qPCR run. Results were reported as the Cq value (the number of cycles taken to reach the fluorescence detection threshold). Negative controls consistently exhibited no detectable fluorescence. Each DNA sample was processed in duplicate, and a sample was considered positive only if there was amplification of each duplicate and the average Cq was less than or equal to 37 cycles. This Cq cut-off value was determined using a standard curve of known *T. gondii* target DNA concentrations. Target DNA was repeatably detected at concentrations of 1×10^{-5} pg/μl at a Cq = 37; lower concentrations were not reliably detected (data not shown). Results were converted to a binary variable (positive or negative) for analysis.

2.6. DNA extraction control

Each cat DNA sample with a negative *T. gondii* qPCR result was amplified using universal eukaryotic primers directed at a highly conserved region of the 18S rRNA gene as a DNA extraction control, to confirm the presence of amplifiable DNA in the sample, as described by Fajardo et al. (2008). Positive (*T. gondii* genomic DNA) and negative controls were included in each reaction. PCR was performed in a thermocycler (Applied Biosystems, Foster City, California, USA). PCR products were visualised using gel electrophoresis on 1.5% agarose gel containing Gel Red® (Biotium, Fremont, California, USA) nucleic acid gel stain.

2.7. Comparison of the two test methods and Bayesian modelling

The overall agreement of the two tests, Cohen's kappa coefficient,

and the prevalence and bias adjusted kappa (PABAK) were calculated using R (R Core Team, 2017) in the epiR statistical package (Stevenson et al., 2018). Cohen's kappa statistic (a measure of test agreement) can be driven down when disease prevalence is extremely high; in these situations, interpretation of PABAK is preferred (Byrt et al., 1993). Although qPCR is a direct test of infection and the MAT is an indirect test, the presence of both bradyzoite tissue cysts (infection) and antibodies is lifelong in cats. Therefore, the two tests were considered comparable measures of infection. The diagnostic sensitivity and specificity of qPCR and MAT, and the true prevalence of *T. gondii* infection were estimated using a Bayesian approach for two conditionally independent tests in one population as described in Branscum et al. (2005). True prevalence is defined as the proportion of animals in a population that are infected with the disease of interest at a given point in time and takes into account the sensitivity and specificity of the diagnostic test used (Thrusfield and Christley, 2018). This is compared to the 'apparent prevalence', which is the proportion of animals from a representative sample of the population that are positive to the diagnostic method used. The prior distribution inputs for test sensitivity and specificity were determined by expert opinion. Three experts in diagnostic parasitology were contacted and the final prior distribution inputs used in the model were the means of values elicited from the experts (Table 1). Published data were not available for the qPCR protocol as this was a novel technique. Only one study has estimated the sensitivity and specificity of the MAT in cats (Macrì et al., 2009). However, comparisons were made to an imperfect reference test (IFAT), which likely overestimated each parameter. Therefore, while considered by the experts as a component of the literature, the estimates from that study were not directly included in the prior distribution inputs for MAT. The prior distribution for true prevalence was based on published data, focusing on those studies reporting *T. gondii* prevalence in feral cats on Australian islands (Adams et al., 2008; Fancourt and Jackson, 2014; O'Callaghan et al., 2005) and expert opinion. The joint posterior distribution of diagnostic sensitivity and specificity of both tests, and true prevalence were obtained using Markov chain Monte Carlo (MCMC) techniques implemented in the WINBUGS© package version 1.4 (Lunn et al., 2000) and R (R Core Team, 2017). The model was run for 40,000 iterations and the first 4,000 samples were discarded as 'burn-in' and chains thinned by a factor to appropriately reduce autocorrelation to < 0.1. The point estimate and 95% credible interval (CrI) for diagnostic sensitivity, specificity, and true prevalence were reported as the median and 2.5% and 97.5% quantiles of the posterior distributions. The tests were assumed to be conditionally independent, because each test was detecting a different manifestation of disease i.e. MAT is an indirect test and detects the host's immune response to infection with *T. gondii* (antibodies) and qPCR detects the organism directly (*T. gondii* DNA). In order to check this theoretical assumption, a sensitivity analysis was run using the model for two dependent tests (Branscum et al., 2005). Sensitivity analyses of each prior distribution was also undertaken. The lower credible limit of each of the priors was decreased by 10% and then by 20%, while keeping all other model inputs constant. Any change of 5% or more in the point estimate produced by the model was considered substantial and is reported in the Results section. Additionally, the prior distributions were plotted against the posterior distributions obtained from the model to allow a visual assessment of the effect of the informative prior distributions on the resulting posterior distributions from the model (Supplementary data, Fig. S2).

2.8. Logistic regression

Regression analyses were carried out using Stata (StataCorp, College Station, Texas, USA). QGIS version 3.8 (QGIS Development Team, 2018) was used to plot trapping locations. To assess risk factors associated with *T. gondii* qPCR positivity in cats, logistic regression models were constructed as guided by a putative causal diagram in the form of a directed acyclic graph (DAG) (Fig. 2). Age on trapping was considered not feasible to accurately and objectively measure for this study population. Weight was considered a rough proxy for age when constructing the putative causal diagram. To be considered as a categorical variable, weight was divided into 4 categories, or 'age classes', based on quantiles in the dataset (Q1 ≤ 1.5 kg, 1.5 > Q2 < = 2.7, 2.7 < Q3 ≤ 3.8, Q4 > 3.8). In order to find the best model fit for the data, weight was also considered as a continuous variable. To determine the most appropriate way to include weight into the analysis as a continuous variable, a Lowess (Locally Weighted Scatterplot Smoothed) curve and a restricted cubic spline were fit to a scatter plot of the log odds of qPCR positivity versus weight in kilograms (Supplementary data, Fig. S1). This indicated that the relationship was nonlinear, and that the most appropriate fit could be achieved by incorporating weight into regression models as a quadratic term centred on 4 kg. To include location data in regression models as a categorical variable, an aerial view (Google Maps™, 2018; OpenLayers Plugin, QGIS) of Phillip Island was used to classify the study area into three different land use types: "Park" was land managed by Phillip Island Nature Parks, wildlife sanctuaries, and other public reserves and parks; "Agricultural" was cleared land, both actively farmed and not currently in use; "Residential" was any area with more than 3-4 adjacent buildings/dwellings (Fig. 1).

Individual models were constructed to explore the relationship between each risk factor and *T. gondii* qPCR positivity separately, conditioning on appropriate covariates, considered here to only be those variables in the minimum adjustment set for estimating the total effect of each variable, as guided by the DAG represented in Fig. 2. For those variables for which there was a statistically significant effect on *T. gondii* qPCR positivity, biologically plausible two-way interactions between risk factors were tested for statistical significance. Comparison of Akaike's information criterion (AIC) between models was used to determine which final model was the best fit for the data.

3. Results

3.1. Demographics of feral cats sampled and Toxoplasma gondii prevalence

A total of 161 feral cats (91 males and 69 females) was collected between July 2016 and December 2017. Body weight ranged from 0.5 to 6.2 kg (mean = 2.7, SD = 1.4). Sex and weight data were not available for one cat. Cats were either live trapped (135), shot (22), hand caught (2), or found as roadkill (1). Capture data was not recorded for one cat. Serum was available from 97 cats. *Toxoplasma gondii* seroprevalence, as detected by MAT, was 91.8% (95% CI 84.6–95.8).

Table 1
Prior distributions for Bayesian estimation of the diagnostic specificity and sensitivity of the modified agglutination test (MAT) and real-time PCR (qPCR) for *Toxoplasma gondii* infection in cats, and true prevalence of infection on Phillip Island (Victoria), determined from expert opinion and relevant available literature.

		Mode	Lower limit 95%	Beta distribution	References informing prior distribution
MAT	Specificity	85.3%	70%	(23.127,4.813)	Expert opinion
	Sensitivity	91.0%	78%	(28.028,3.673)	Expert opinion
qPCR	Specificity	99.7%	80%	(13.737,1.038)	Expert opinion
	Sensitivity	94.3%	82%	(28.219,2.645)	Expert opinion
True prevalence		89.8%	76%	(26.111, 3.852)	Adams et al., 2008; Fancourt and Jackson, 2014; O'Callaghan et al., 2005;Expert opinion

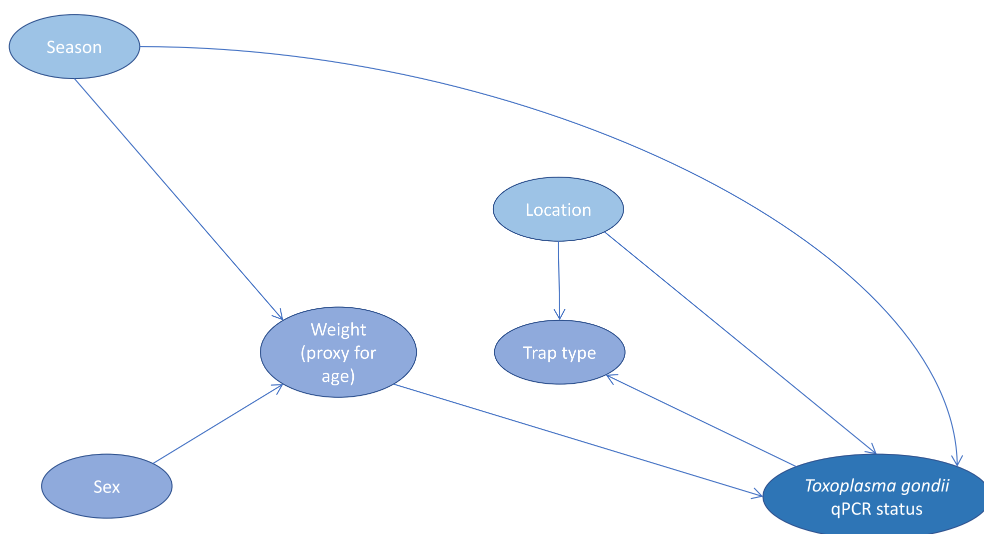

Fig. 2. Directed acyclic graph (DAG) showing the putative causal framework for *Toxoplasma gondii* infection in feral cats on Phillip Island, used to inform the approach to logistic regression modelling of the relationship between risk factors and qPCR positivity.

1) Season; likely to affect age of cats sampled. Cats are seasonal breeders and therefore young animals are more likely to be trapped during certain seasons. Season may also affect weight due to seasonal variation in prey availability. Additionally, season was postulated to have a direct effect on *T. gondii* infection status, as has been noted in other studies (e.g. Afonso et al., 2013).
2) Weight; considered here to be a rough proxy for age and therefore increasing weight (age) likely to increase the probability of being *T. gondii* positive due to the cumulative increase in opportunities for exposure.
3) Location; prevalence of infection in intermediate hosts may vary across locations and therefore the probability of infection in feral cats may vary with location. Location may determine the type of trapping most likely employed e.g. shooting in remote areas versus trapping in residential/urban areas.
4) Trap type; *T. gondii* positive cats may be more likely to be caught via certain methods e.g. *T. gondii*-infected cats may have reduced inhibition compared to uninfected animals and therefore may be more likely to enter a trap.
5) Sex; in general, male cats are larger than female cats and therefore may have a direct influence on weight.

Toxoplasma gondii qPCR results were available for all cats (n = 161). *Toxoplasma gondii* apparent prevalence, as detected by qPCR, was 79.5% (95% CI 72.6–85.0). Capture locations were distributed across most of the island and *T. gondii* qPCR positive individuals were widespread (Fig. 1). DNA extraction controls confirmed amplifiable eukaryotic DNA was present in all *T. gondii*-negative samples (data not shown).

3.2. Evaluation of the modified agglutination test and real-time PCR as diagnostic tests

The overall agreement between MAT and qPCR was 87.6% (Table 2). Given the extremely high prevalence of *T. gondii* infection in the present study, PABAK was calculated in preference to Cohen's kappa. PABAK was 0.753 (95% CI 0.588–0.869), indicating substantial agreement between the two tests (Byrt et al., 1993).

Bayesian estimations of the diagnostic test parameters for qPCR and MAT, and the estimated true prevalence of *T. gondii* infection in the population are outlined in Table 3. On sensitivity analysis, no substantial changes were observed in the point estimates for the true prevalence of *T. gondii*, the sensitivity and specificity of the qPCR method, or the sensitivity of the MAT. When the lower credible limit of the prior distribution of the specificity of the MAT (SpMAT) was lowered to 50%, the point estimate of the specificity was reduced to 74.3% (95% CrI 44.5–95.7). Visual assessment confirmed that posterior distributions for all parameters except SpMAT were informed by the data, rather than overly influenced by the prior distributions. The plot of prior and posterior distributions of SpMAT suggested that this parameter may have been strongly influenced by the prior distribution inputs. When the data and prior distributions were analysed using the dependent tests model, the 95% CrI for the correlation terms crossed zero, satisfying the assumption that the tests were conditionally independent and justifying the use of the independent test model.

Table 2
Contingency table comparing modified agglutination test (MAT) and *Toxoplasma gondii* qPCR results for feral cats trapped on Phillip Island.

	MAT positive	MAT negative
qPCR positive	78	1
qPCR negative	11	7

Table 3
Sensitivity and specificity of the *Toxoplasma gondii* qPCR protocol and the modified agglutination test (MAT) and true prevalence of infection in feral cats on Phillip Island as determined by Bayesian latent class analysis.

		Point estimate (95% Credible interval)
MAT	Sensitivity	96.2% (91.8–98.8)
	Specificity	82.1% (64.9–93.6)
qPCR	Sensitivity	90.1% (83.6–95.5)
	Specificity	96.0% (82.1–99.8)
True prevalence		90.3% (83.2–95.1)

3.3. Risk factors associated with Toxoplasma gondii qPCR positivity

Logistic regression was performed using the qPCR dataset only due to its greater sample size. Weight, as a proxy for age, was modelled as a continuous and a categorical variable. When weight was included as a categorical variable, the interaction term between weight and season could not be modelled due to numerical issues associated with sample size. Akaike's information criterion was compared between three possible models for the total effect of weight on *T. gondii* qPCR positivity

Table 4
Comparison of Akaike's information criterion (AIC) for putative logistic regression models estimating the total effect of weight on *Toxoplasma gondii* qPCR positivity in feral cats.

	Variables included	AIC
Model 1	Weight (categorical), Season	160.62
Model 2	$Weight^2$, Season	157.20
Model 3	$Weight^2$, Season, Weight*Season	155.47

Weight (categorical) = categorical term, four categories based on data quantiles.
$Weight^2$ = continuous quadratic term centred on 4 kg, of the form (weight - 4 kg)^2.

Table 5
Final multivariable logistic regression model for the total effect of weight on *Toxoplasma gondii* qPCR positivity in feral cats on Phillip Island (Victoria) from July 2016 to December 2017.

	Odds Ratio	95% Confidence Interval	P > |z|
Season [a]			
Summer	1.25	0.15–10.13	0.837
Winter	0.71	0.13–3.78	0.686
Spring	1.22	0.13–11.64	0.863
Weight [b]	0.77	0.62–0.94	**0.011**
Season*Weight interaction term [a]			
Summer	1.10	0.80–1.51	0.566
Winter	1.41	1.04–1.90	**0.027**
Spring	4.68	0.11–201.70	0.421

[a] - compared to Autumn as the reference season. The minimum adjustment set for the total effect of Season did not include any other variables and estimates of this effect are presented in Supplementary Material (Table S1).
[b] - incorporated into the model as quadratic term centred on 4 kg of the form (weight - 4 kg)^2.

(Table 4). Model 3, which included weight as a continuous variable and the interaction term between weight and season, had the lowest AIC and was chosen as the final model. Outputs from Model 3 are presented in Table 5. Outputs from individual models constructed to estimate the total effect of each risk factor considered, as guided by the putative causal diagram, are presented in Supplementary materials (Table S1). To aid in interpretation of the interaction term, the outputs from the multivariable logistic regression model for the total effect of weight were plotted as predicted probabilities of qPCR positivity in cats versus weight in kilograms, for each season (Fig. 3).

4. Discussion

The apparent prevalence of *T. gondii* infection in the feral cat population on Phillip Island, based on qPCR, was 79.5% and seroprevalence was 91.8%. There was substantial agreement between the two tests, and each instance of test disagreement is discussed further as follows. MAT-negative, qPCR-positive samples are consistent with recent infection, as a detectable IgG response develops between 14- and 21-days post-infection (Dubey et al., 1995). Therefore, *T. gondii* tachyzoites may be detected by qPCR prior to the presence of IgG antibodies. Prozone phenomenon (lack of agglutination due to extremely high antibody concentration) was accounted for by testing each sample in serial dilutions and is not considered likely. MAT-positive, qPCR-negative samples may be explained by either MAT false positives or qPCR false negatives. To minimise false negative results, the qPCR protocol in this study incorporated a large quantity of tissue (5 g) and included the optimal bradyzoite predilection sites in cats. Furthermore, the present study indicated that the diagnostic specificity of the qPCR protocol was much higher than the MAT, suggesting that MAT false positives may be more likely. These false positives may be explained by serological cross-reactivity with closely related apicomplexans, such as *Hammondia hammondi* (Dubey and Sreekumar, 2003; Riahi et al., 1998). PCR cross-reactivity between *H. hammondi* and *T. gondii* is not applicable in this study as *H. hammondi* does not form tissue cysts in cats (Dubey and Sreekumar, 2003). Thus, the key advantage of this qPCR protocol is its superior specificity for detecting *T. gondii* infection in cats when compared with the MAT. The main disadvantage to this qPCR method is that it cannot be used for ante-mortem epidemiological screening. However, this is unlikely to be a significant issue in studies investigating *T. gondii* epidemiology in feral animal populations. For future studies, the authors recommend the following approach: initial screening of samples using the MAT due to its high sensitivity, faster throughput, and lower cost. MAT positives should then be confirmed as *T. gondii* infected with qPCR to detect any false positives.

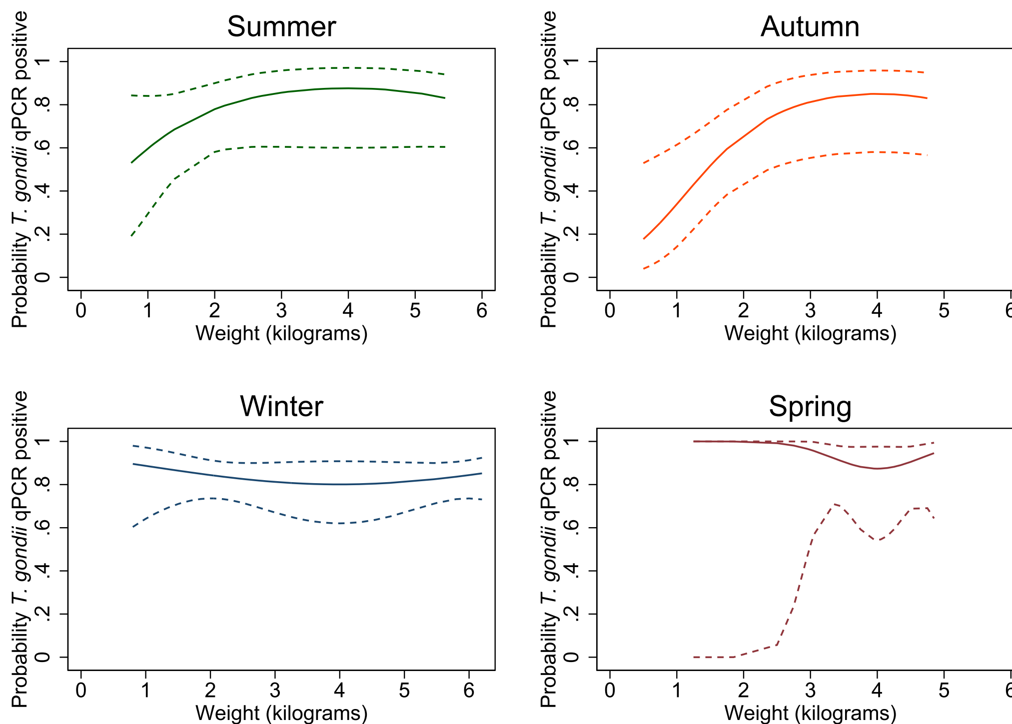

Fig. 3. Predicted lines of fit for the multivariable logistic regression model plotted as probability of *Toxoplasma gondii* qPCR positivity in feral cats on Phillip Island (Victoria) versus body weight for each season. Dashed lines show 95% confidence intervals.

The Bayesian models used in the present study were robust on sensitivity analysis, indicating that the results were primarily driven by the data, rather than being overly influenced by the prior distribution inputs. Only one parameter, the specificity of the MAT, varied substantially on sensitivity analysis. This suggests this parameter may have been overly informed by the priors, rather than the data. Due to time and cost limitations in this study, the final sample size was smaller than the desired sample size required to estimate specificity of the MAT. This is likely responsible for the variability and reduced precision in this parameter. Regardless, significant variation in the point estimate only occurred when the lower limit of the prior distribution was lowered to 50%. Based on published diagnostic specificities for this test in other species, this lower limit was considered very unlikely. Therefore, although the specificity of the MAT should be interpreted with caution, the results of the Bayesian analysis in this study are considered valid.

The final logistic regression model for the total effect of weight on the probability of *T. gondii* infection indicated an interaction between weight and season. As seen in Fig. 3, the probability of being *T. gondii* qPCR positive increased with increasing weight in summer and autumn, reaching a plateau at weights greater than 4 kg. If weight is considered a rough proxy for age then, in summer and autumn, these results suggest that, as expected, *T. gondii* infection risk increases with age. However, in winter and spring, the probability of *T. gondii* infection remained at 80% or above, irrespective of weight. This result may be explained by a greater proportion of younger, lower weight, animals being trapped in autumn and summer. However, low weight cats were also trapped in winter and *T. gondii* infection prevalence was very high in these animals, suggesting that the interaction between weight and season may be more complex. Oocyst environmental survival is greatest when the weather is cool and wet (Frenkel et al., 1975; Yilmaz and Hopkins, 1972), and high oocyst survival will lead to increased transmission to intermediate hosts and a subsequent increase in *T. gondii* prevalence in cats via predation. Studies elsewhere have also found associations between season and/or climactic factors and *T. gondii* infection in cats, e.g. *T. gondii* incidence risk increased with increasing rainfall (Afonso et al., 2010) and seroprevalence was positively associated with milder, wetter winters in Europe (Afonso et al., 2013). Other factors may affect the frequency of *T. gondii* transmission, such as the relative abundance of intermediate hosts, variable oocyst exposure across different prey taxa, and feline dietary preferences. These factors may also vary with season, and therefore may help to explain the relationship between season and *T. gondii* infection prevalence in feral cats. For example, feline dietary preferences have been shown to be affected by both temperature and rainfall and thus will fluctuate with season (Doherty et al., 2015; Kirkwood et al., 2005).

5. Conclusion

The estimated true prevalence of *T. gondii* infection in the feral cat population on Phillip Island was found to be very high, indicating that toxoplasmosis may pose a significant risk to native wildlife inhabiting the island. Furthermore, *T. gondii* infection risk in cats was found to be associated with season. However, this association is complex and further investigations are required to better understand the significance of season in the epidemiology of *T. gondii* in the Australian landscape.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2020.05.006.

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